Bioresource Technology 108 (2012) 295–299

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Effect of exogenous electron shuttles on growth and fermentative metabolism in *Clostridium* sp. BC1

Venkata Nancharaiah Yarlagadda [a,b,*], Ashutosh Gupta [a], Cleveland J. Dodge [a], Arokiasamy J. Francis [a,c]

[a] Environmental Sciences Department, Brookhaven National Laboratory, Upton, NY 11973, USA
[b] Biofouling and Biofilm Processes Section, Water and Steam Chemistry Division, Bhabha Atomic Research Centre, Kalpakkam 603102, Tamil Nadu, India
[c] Division of Advanced Nuclear Engineering, POSTECH, Pohang, South Korea

ARTICLE INFO

Article history:
Received 12 October 2011
Received in revised form 8 December 2011
Accepted 8 December 2011
Available online 16 December 2011

Keywords:
Biobutanol
Bioethanol
Clostridium
Electron shuttles
Methyl viologen

ABSTRACT

In this study, the influence exogenous electron shuttles on the growth and glucose fermentative metabolism of *Clostridium* sp. BC1 was investigated. Bicarbonate addition to mineral salts (MS) medium accelerated growth and glucose fermentation which shifted acidogenesis (acetic- and butyric-acids) towards solventogenesis (ethanol and butanol). Addition of ferrihydrite, anthraquinone disulfonate, and nicotinamide adenine dinucleotide in bicarbonate to growing culture showed no significant influence on fermentative metabolism. In contrast, methyl viologen (MV) enhanced ethanol- and butanol-production by 28- and 12-fold, respectively with concomitant decrease in hydrogen, acetic- and butyric-acids compared to MS medium. The results show that MV addition affects hydrogenase activity with a significant reduction in hydrogen production and a shift in the direction of electron flow towards enhanced production of ethanol and butanol.

1. Introduction

Electron shuttle (ES) compounds that cycle between oxidized and reduced states in multiple redox reactions play a vital role in bacterial metabolism. Exogenous supplements of ESs can create alternate electron flows, thereby affecting the fermentation pattern and biofuel production. Thus, exogenous ESs are added to accelerate electron transport and to modify the energy metabolism and physiology of microorganisms. ESs such as neutral red, methylene blue, methyl viologen (MV) and benzyl viologen were used to shift fermentative metabolism towards alcohol production (Datta and Zeikus, 1985; Peguin and Soucaille, 1995; Steinbusch et al., 2010; Tashiro et al., 2007). Addition of reduced anthraquinone disulfonate (AQDS) altered the fermentative pattern of *Clostridium beijerinckii* by increased H_2 production (Hatch and Finneran, 2008). Although the exact mechanism whereby ESs regulate the fermentative pathway is not fully understood, adding them to bacterial cultures is an attractive strategy for shifting the direction of electron flow from generating organic acids to alcohols (ethanol and butanol) and hydrogen (Reimann et al., 1996).

Anaerobic fermentative bacteria belonging to the genus *Clostridia* are of special interest in biofuel production from lignocellulosic biomass (Hess et al., 2011) due to their innate ability to ferment 5 and 6 carbon sugars. *Clostridium* sp. BC1 (ATCC 53464) (henceforth referred to as BC1) isolated from an acidic metal contaminated site, has demonstrated efficient reduction of several redox-active metals (i.e. U(VI), Pu(IV), Tc(VII), Fe(III) and Mn(IV)) in mineral salts medium (Francis et al., 2008). BC1 was found to be a rapid and efficient glucose fermenter compared to other *Clostridia* strains, *Clostridium acetobutylicum* (ATCC 19403), *Clostridium sphenoides* (ATCC 19403), and *Clostridium pasteurianum* (ATCC 7040) (Gao and Francis, 2008). It was hypothesized that the electron shuttle compounds such as ferrihydrite ($Fe(OH)_3$), nicotinamide adenine dinucleotide (NADH), AQDS and MV cause a significant shift in the metabolic flow in the carbon metabolism of the metal reducing bacterium BC1 from acidogenesis to soleventogenesis resulting in enhanced ethanol and butanol production. $Fe(OH)_3$ is a widespread ferric hydroxide and is an alternate electron acceptor under anaerobic conditions. AQDS is widely used as a humic acid analogue for investigating the role of quinone based ESs. NADH is the natural electron carrier present in the cells. MV has reduction potential closer to that of ferredoxin, which mediates electron transfer in a range of metabolic reactions. To understand the effect of ES addition on the fermentative metabolism of BC1, we monitored the growth, glucose consumption and production of organic acids (acetic-, butyric- and lactic-acids), alcohols (ethanol and butanol) and gasses (H_2 and CO_2).

* Corresponding author at: Biofouling and Biofilm Processes Section, Water and Steam Chemistry Division, Bhabha Atomic Research Centre, Kalpakkam 603102, Tamil Nadu, India. Tel.: +91 44 27480500x23342; fax: +91 44 27480097.
E-mail addresses: venkatany@gmail.com, yvn@igcar.gov.in (V.N. Yarlagadda).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.12.040

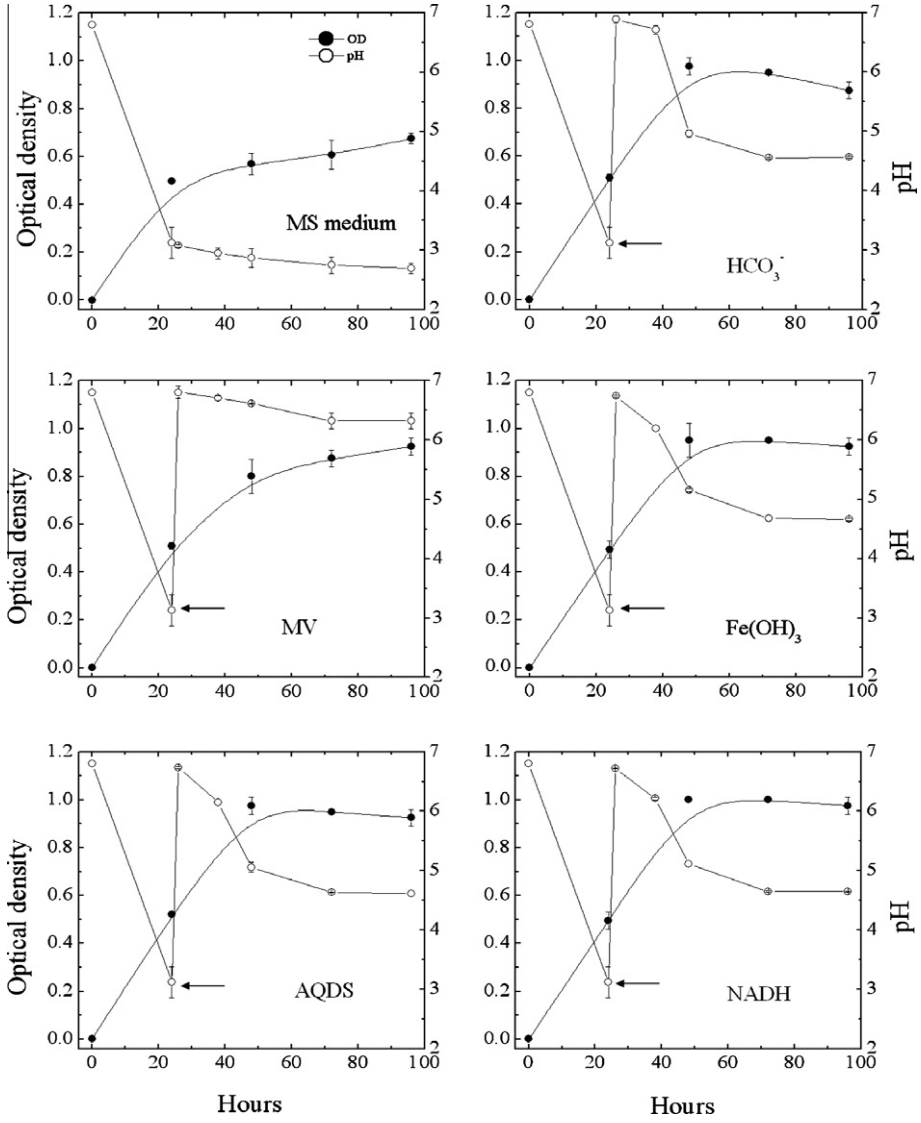

Fig. 1. Growth (OD 600 nm) and medium pH profiles during batch fermentation in mineral salts (MS) medium by *Clostridium* sp. BC1. Arrow indicates the addition of bicarbonate and electron shuttles prepared in bicarbonate at 24 h after inoculation. Error bars represent ±1SD. Error bars are not seen in some data points due to minimal variation between duplicates.

2. Methods

2.1. Electron shuttles

MV, NADH, and AQDS were obtained from Sigma–Aldrich. ES solutions were prepared by dissolving 100 mg each of MV, NADH and AQDS in 40 mL of 1 M sodium bicarbonate. $Fe(OH)_3$ was prepared as described previously (Dodge et al., 2002). ES solutions were purged with nitrogen gas for 10 min and stored under nitrogen atmosphere in serum bottles. HCO_3^- solution was also stored under similar conditions.

2.2. Culture and growth conditions

BC1 was grown in the mineral salts (MS) medium containing 1% (w/v) glucose as described previously (Nancharaiah and Francis, 2011). The medium was pre-reduced by boiling for 10 min while purging with ultra-high-purity nitrogen gas dispensed as 40-mL aliquots into 60-mL serum bottles in an anaerobic chamber (Coy laboratory products, USA), fitted with butyl rubber stoppers, crimp sealed with aluminum caps and autoclaved. The culture was maintained by inoculating the MS medium with 1-mL of log phase culture and incubating at 26 °C.

2.3. Growth and fermentative metabolism

The serum bottles containing pre-reduced sterile MS medium were inoculated with 1-mL of a log-phase culture ($OD_{600\ nm}$ = 0.4) and incubated at 26 °C. A 1.5-mL of HCO_3^- or ES in HCO_3^- was added to the culture after 24 h of growth. The final concentration of MV, NADH, AQDS and $Fe(OH)_3$ in the culture was 0.36, 0.13, 0.23 and 0.56 mM, respectively. All the treatments were performed in triplicates. Periodically, 5 mL of the culture was withdrawn and analysed for optical density, pH, organic acids and alcohols.

2.4. Chemical analysis

At periodic intervals, total gas production was determined by measuring the head space for pressure using a pressure gauge connected to syringe needle. The CO_2 and H_2 concentrations in the

headspace were measured by gas chromatography using a Gow Mac series 580 gas chromatograph under isothermal mode using thermal conductivity detector. The culture medium pH was determined using a Beckman Φ350 pH meter with a Beckman 511275-AB combination electrode. The culture sample was then filtered through a 0.45 μm Millex filter, and glucose, alcohols and organic

Table 1
Comparison of the production of various fermentation products by *Clostridium* sp. BC1 grown in mineral salts (MS) medium with and without bicarbonate and ESs. The metabolite concentrations were determined after 96 h of incubation.

Substrate or product (mM)	Medium					
	MS	$NaHCO_3$	$NaHCO_3$			
			$Fe(OH)_3$	AQDS	NADH	MV
Glucose utilized	32.3	54.9	55.0	55.0	55.0	51.5
Total organic acids	41.7	75.7	75.3	77.9	74.6	36.7
Acetic acid	25.5	62.8	63.1	64.6	62.4	29.2
Butyric acid	15.5	12.2	11.5	12.6	11.4	1.0
Lactic acid	0.7	0.7	0.7	0.7	0.8	6.5
Total solvents	2.8	17.4	18.5	17.4	19.7	48.4
Butanol	2.0	12.9	13.5	12.8	14.1	24.9
Ethanol	0.8	4.5	5.0	4.6	5.6	23.5

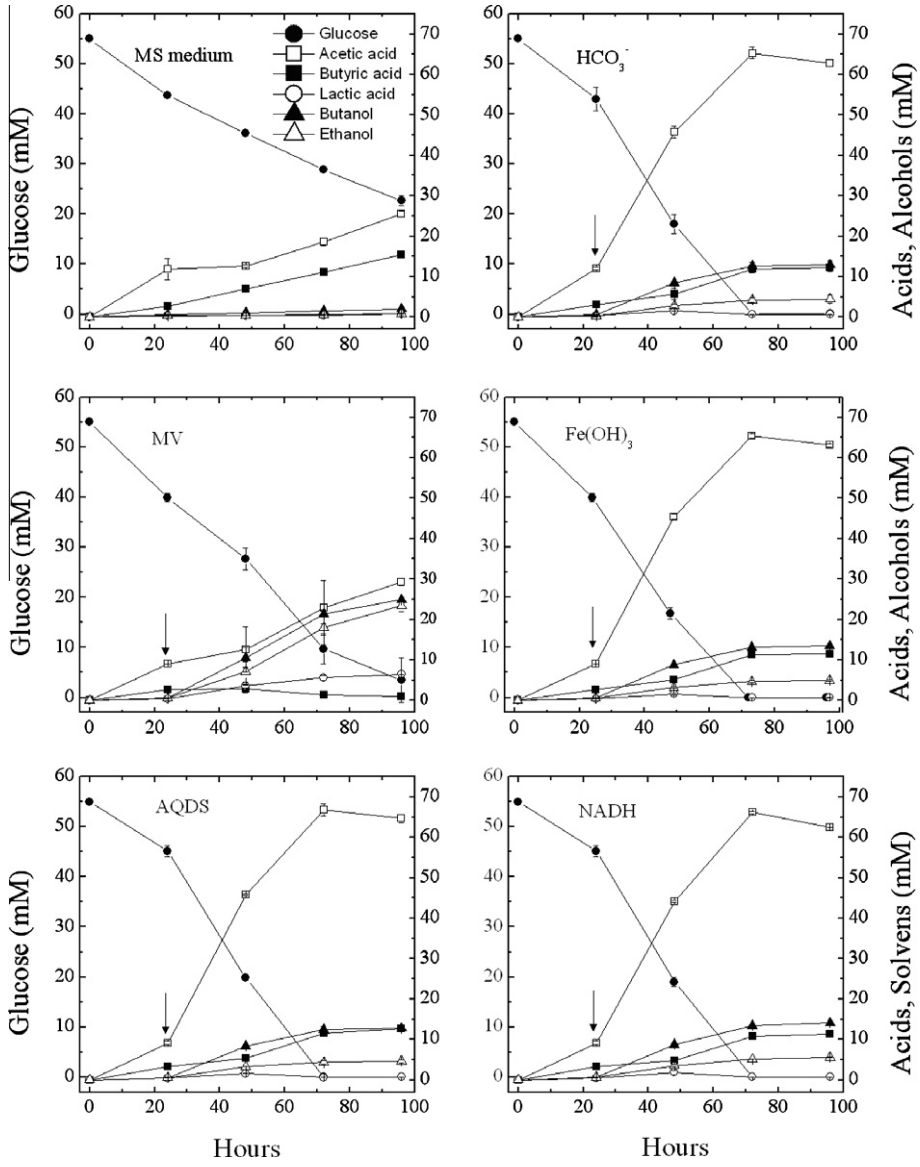

Fig. 2. Production of metabolites (acids and alcohols) during batch fermentation by *Clostridium* sp. BC1 in mineral salts (MS) medium. Arrow indicates the addition of bicarbonate and ES in bicarbonate at 24 h after inoculation Error bars represent ±1SD. Error bars are not seen in some data points due to minimal variation between duplicates.

acids were analyzed by high pressure liquid chromatography using a Shimadzu LC-10AS liquid chromatography fitted with a Bio-Rad HPX-87H column. A diluted sulphuric acid (0.003 N) was used as mobile phase at a flow rate of 0.7 mL min^{-1}. Glucose, alcohols and organic acids were determined using a refractive index and a UV–vis detector, respectively.

3. Results and discussion

3.1. Effect of bicarbonate and electron shuttles on BC1 growth

Fig. 1 shows the growth of BC1 in MS medium and the accompanying changes in the medium pH during batch fermentation. BC1 growth pattern was characterized with distinct, rapid exponential- and stationary-phases. The rapid exponential growth of BC1 during the first 24 h entailed a significant drop in the medium pH to ~3. Adding MV at the time of inoculation, completely inhibited BC1 growth (data not shown). ESs were added to BC1 after 24 h of inoculation so as to minimize inhibitory effect on growth. Furthermore, HCO_3^- was added in order to raise the pH of the culture medium, which could result in better growth. Addition of HCO_3^- or ESs facilitated the rapid growth of BC1 by raising medium pH to ~7.0. Increased growth of BC1 was corroborated with a drop in medium pH, with the final pH reaching ~4.5 (Fig. 1). The maximum cell growth due to HCO_3^-, $Fe(OH)_3$, AQDS, or NADH addition was almost similar. Overall, growth pattern and medium pH profile was similar with added HCO_3^- and in all ES additions, except that, following addition of MV. Since all ESs also contained HCO_3^- it can be concluded that $Fe(OH)_3$, AQDS and NADH had no effect on BC1 growth, while the observed effect was most likely due to HCO_3^- addition. In MV addition, the medium pH was stabilized at ~6.3, about 1.5 units higher compared to HCO_3^- and other ESs. The less pH drop in MV addition was most likely due to metabolic perturbation and fermentation product distribution. The culture medium pH is recognized as an important factor influencing fermentative metabolism (Li et al., 2011; Zhu and Yang, 2004). In general, neutral pH promotes growth and accelerates the fermentative metabolism. The pH of the MS medium with MV addition remained near neutral during the fermentation, while it was ~4.5 in the presence of HCO_3^-, NADH, $Fe(OH)_3$, or AQDS (Fig. 1). In order to discern pH effect on growth and alcohol production, BC1 was grown in phosphate-buffered MS (PMS) medium. With MV addition, the pH of PMS medium was stabilized at ~5 as compared to ~4.7 in PMS medium control (Supplementary Fig. S1). From the above discussion, it can be concluded that electron shunting in BC1 was primarily driven by MV.

3.2. Organic acid and alcohol production

In the MS medium (control), BC1 fermented glucose to the following (mol/mol glucose): acetate, 0.79; butyrate, 0.48; lactate, 0.02; butanol, 0.06; ethanol, 0.03 (Table 1). HCO_3^- addition caused a significant increase in acetate, butanol and ethanol levels. The butanol and ethanol levels were increased by 7- and 6-fold respectively, compared to MS medium. While the butyric-acid levels were marginally decreased, lactic-acid levels were unaltered. Overall, alcohol production after adding either $Fe(OH)_3$, AQDS or NADH resembled that occasioned by HCO_3^-; after all these treatments, butanol was the major alcohol produced and ethanol the minor one. Hence, the addition of $Fe(OH)_3$, AQDS or NADH had minimal affect on ethanol and butanol levels. Addition of MV, however, lowered the production of acetic- and butyric-acid, and enhanced butanol and ethanol yields (Fig. 2). Among the ESs tested, only MV significantly enhanced ethanol and butanol production by 28- and 12-fold, respectively, compared to MS medium (control). The increase in ethanol and butanol levels were 5- and 2-fold, respectively compared to HCO_3^-. This difference in product distribution with different ESs could be attributed to the differences in the reduction potentials of ESs used in this study. Since MV has the closest reduction potential to that of ferredoxin, the natural electron carrier in *Clostridia*, only MV could possibly compete with ferredoxin and cause changes in carbon and electron flow. The yields of butanol (0.48) and ethanol (0.46) with MV addition were eight and eighteen times higher than those obtained in MS medium. The alcohol yields obtained in this study are higher than the increase observed due to addition of 1 mM of MV to *C. acetobutylicum* culture (Peguin and Soucaille, 1995). Unlike *C. acetobutylicum*, addition of MV to BC1 increased the production of both butanol and ethanol. MV addition to PMS medium also increased both butanol and ethanol levels by decreasing acetic- and butyric-acids (Supplementary Fig. S2, and Table S1). Lactic-acid levels were increased by MV addition to both MS and PMS medium. Moreover, BC1 was unable to utilize the lactate produced, unlike *C. acetobutylicum*.

3.3. Total gas and hydrogen production

Total gas production was similar in HCO_3^-, $Fe(OH)_3$, AQDS or NADH addition and 50–60% higher compared to MS medium and MV addition (Supplementary Fig. S3). HCO_3^- addition increased CO_2 production from 1.4 to 3.46 mmol without significantly altering CO_2 levels (Fig. 3). The increase in CO_2 levels could be attributed to enhanced growth and glucose utilization and due to the HCO_3^- itself, which releases CO_2 in the process of controlling the pH. MV addition caused a drastic decrease in H_2 evolution with a negligible influence on CO_2 levels (Fig. 3). H_2 evolution was nearly 85% less after MV addition as compared to HCO_3^- addition. There was a 4-fold increase in the CO_2:H_2 ratio due to MV addition as compared to HCO_3^- addition. In the phosphate-buffered MS medium the CO_2 and H_2 ratio increased by ~4.5-fold upon the addition

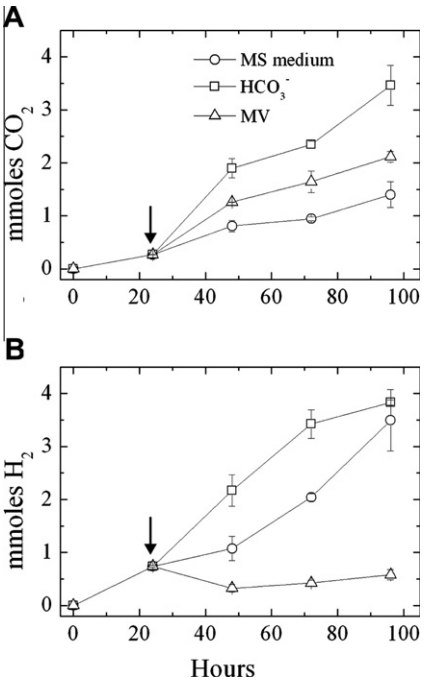

Fig. 3. Production of carbon dioxide (A) and hydrogen (B) during glucose fermentation in mineral salts (MS) medium by *Clostridium* sp. BC1. Arrows indicates the addition of bicarbonate or MV prepared in bicarbonate at 24 h after inoculation. Error bars represent ±1SD.

of MV (Supplementary Fig. S4), suggesting that the effect of MV on BC1 is independent of medium pH. Overall, MV addition decreased the H_2 evolution significantly in MS or PMS medium, with the complete stoppage of H_2 production after MV addition.

Because of reduction potential similarity, only MV and not $Fe(OH)_3$, AQDS or NADH can possibly compete with ferredoxin in pyruvate:ferredoxin oxidoreductase reaction. MV was immediately reduced (blue color) when added to BC1 (Supplementary Fig. S5). This reduced MV would compete with electron flow to hydrogenase by creating an alternate electron flow thereby generate NADP(H) (Supplementary Fig. S6). This mechanism can lead to lower H_2 production and higher NADP(H) in the culture medium, which is needed for production of reduced metabolites (butanol, ethanol). The data implicate direct involvement of MV in interfering with electron flow to H_2 by acting as an artificial electron carrier and directing the electron flow to production of butanol, ethanol, and lactate. The yields of these reduced products can be improved if the production of organic acids (acetic- and lactic-acids) can be further minimized by imposing selectivity towards ethanol or butanol by understanding pathways via genomic sequencing (Paul et al., 2010) and pathway engineering (Huang et al., 2010).

4. Conclusions

Clostridium sp. BC1 appears to be distinctly different from the well studied *Clostridia* strains in terms of rapid glucose fermentation, its response to MV in the elevation of both ethanol and butanol yields. HCO_3^- addition accelerated the growth and fermentative metabolism. $Fe(OH)_3$, AQDS, or NADH addition had negligible effect on electron flow in BC1. MV diverted the electron flow towards the production of reduced metabolites, ethanol and butanol. The effect of MV addition on *Clostridium* sp. BC was characterized by (i) decreased acetic- and butyric-acid production, (ii) decreased hydrogen production, (iii) enhanced lactic-acid production, and (iv) enhanced ethanol- and butanol-production.

Acknowledgements

This research was supported in part by Brookhaven National Laboratory, Laboratory Directed Research and Development (LDRD) project, US Department of Energy under contract No. DE-AC02-98CH10886, and by WCU (World Class University) program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R31-30005). YVN acknowledges American Society for Microbiology (ASM) for 2009 Indo-US Visiting Research Professorship Award.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.12.040.

References

Datta, R., Zeikus, J.G., 1985. Modulation of acetone–butanol–ethanol fermentation by carbon monoxide and organic acids. Appl. Environ. Microbiol. 49, 522–529.
Dodge, C.J., Francis, A.J., Gillow, J.B., Halada, G.P., Eng, C., Clayton, C.R., 2002. Association of uranium with iron oxides typically formed on corroding steel surfaces. Environ. Sci. Technol. 36, 3504–3511.
Francis, A.J., Dodge, C.J., Gillow, J.B., 2008. Reductive dissolution of Pu(IV) by *Clostridium* sp. under anaerobic conditions. Environ. Sci. Technol. 42, 2355–2360.
Gao, W., Francis, A.J., 2008. Reduction of uranium(VI) to uranium(IV) by *Clostridia*. Appl. Environ. Microbiol. 74, 4580–4584.
Hess, M., Sczyrba, A., Egan, R., Kim, T.-W., Chokhawala, H., Schroth, G., Luo, S., Clark, D.S., Chen, F., Zhang, T., Mackie, R.I., Pennacchio, L.A., Tringe, S.G., Visel, A., Woyke, T., Wang, Z., Rubin, E.M., 2011. Metagenomic discovery of biomass-degrading genes and genomes from cow rumen. Science 331, 463–467.
Hatch, J., Finneran, K., 2008. Influence of reduced electron shuttling compounds on biological H_2 production in the fermentative pure culture *Clostridium beijerinckii*. Curr. Microbiol. 56, 268–273.
Huang, H., Liu, H., Gan, Y.-R., 2010. Genetic modification of critical enzymes and involved genes in butanol biosynthesis from biomass. Biotechnol. Adv. 28, 651–657.
Li, S.Y., Srivastava, R., Suib, S.L., Li, Y., Parnas, R.S., 2011. Performance of batch, fed-batch, and continuous A–B–E fermentation with pH-control. Bioresour. Technol. 102, 4241–4250.
Nancharaiah, Y.V., Francis, A.J., 2011. Alkyl-imidazolium ionic liquids affect the growth and fermentative metabolism of *Clostridium* sp. Bioresour. Technol. 102, 6573–6578.
Paul, D., Austin, F.W., Arick, T., Bridges, S.M., Burgess, S.C., Dandass, Y.S., Lawrence, M.L., 2010. Genome sequence of the solvent-producing bacterium *Clostridium carboxidivorans* strain P7. J. Bacteriol. 192, 5554–5555.
Peguin, S., Soucaille, P., 1995. Modulation of carbon and electron flow in *Clostridium acetobutylicum* by iron limitation and methyl viologen addition. Appl. Environ. Microbiol. 61, 403–405.
Reimann, A., Biebl, H., Deckwer, W., 1996. Influence of iron, phosphate and methyl viologen on glycerol fermentation of *Clostridium butyricum*. Appl. Microbiol. Biotechnol. 45, 47–50.
Steinbusch, K.J.J., Hamelers, H.V.M., Schaap, J.D., Kampman, C., Buisman, C.J.N., 2010. Bioelectrochemical ethanol production through mediated acetate reduction by mixed cultures. Environ. Sci. Technol. 44, 513–517.
Tashiro, Y., Shinto, H., Hayashi, M., Baba, S.-I., Kobayashi, G., Sonomoto, K., 2007. Novel high-efficient butanol production from butyrate by non-growing *Clostridium saccharoperbutylacetonicum* N1–4 (ATCC 13564) with methyl viologen. J. Biosci. Bioeng. 104, 238–240.
Zhu, Y., Yang, S.T., 2004. Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. J. Biotechnol. 110, 143–157.